IDENTIFICATION AND EMBRYONIC EXPRESSION OF A HIGHLY CONSERVED *MEIS*-LINKED GENE

A Thesis
by
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Submitted to the Graduate School
Appalachian State University
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

August 2012
Department of Biology

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FOREWORD

This thesis manuscript and the figures, tables, and references herein were formatted according to the author instructions for manuscript submission to the peer-reviewed journal *Developmental Biology*, the official journal of the Society for Developmental Biology, published by Elsevier.

ABSTRACT

IDENTIFICATION AND EMBRYONIC EXPRESSION OF A HIGHLY CONSERVED *MEIS*-LINKED GENE

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Using a comparative genomics approach we have identified a novel and previously undescribed gene in zebrafish, *zgc:154061*, which we have temporarily named *meis2 linked gene* (*m2lg*). This gene is located directly downstream of the zebrafish *meis2.2* gene. We have identified putative orthologs of this gene in all animals for which publicly available genome data is available. *M2lg* and its vertebrate orthologs are organized in a convergently transcribed manner with respect to the *Meis2* gene in all species we have examined (*meis2.2* in zebrafish). During zebrafish development, transcripts of *m2lg* are observed in every cell of the embryo from the earliest stage through the shield stage indicating this gene is a maternal transcript since its expression precedes the activation of the zygotic genome at the midblastula transition. Expression of *m2lg* gradually decreases from its peak value at 0 hours past fertilization (hpf) until 8 hpf and then is observed to be activated again at 12 hpf as determined by quantitative real time polymerase chain reaction (PCR). This later expression is observed throughout the neural tube before becoming restricted to the retina and tectum opticum by 48 hpf. Using an antibody raised against a peptide portion of the predicted protein product of *m2lg* in New Zealand white rabbit, it has

been shown that the gene is translated into protein within the developing embryo and that it is expressed at various stages throughout development. Western blots show that the protein is expressed as early as 2 hpf and is present in significant amounts until 12 hpf. Immunohistochemistry on 48 hpf zebrafish embryo cross-sections show that the protein is present and is highly localized to the retinal area and the optic nerve.

DEDICATION

To my younger sister, Catherine Cochrane, for being by my side during every step of my life and being my best friend in the world, and to my parents Jeff Cochrane and Nancy Cochrane who have given me unconditional support and love.

ACKNOWLEDGMENTS

The work that I have done in my years at Appalachian State University would not have been possible without the aid I have received during my time here. I would first like to extend my gratitude towards the Appalachian State University Office of Student Research, the Appalachian State University Graduate Student Association Senate, the Cratis D. Williams Graduate School, the Wayne Richardson Fellowship, and the Sigma Xi Grant in Aid of Research Program for all of the research funding support I have received during my years of graduate school. I would also like to thank the Appalachian State University Graduate Research Associate Mentoring Award for providing extra funding assistance and allowing me to focus solely on my research for an extended period.

I owe much appreciation to Gary Walker for giving me the opportunity to attend graduate school and for his significant help along the way, as well as Ece Karatan for not only assisting in graduate school affairs but also providing invaluable feedback for many of my protocols and troubleshooting worries. To Betsy Harris I will forever be grateful for her instruction and guidance in teaching Introductory Biology and for so many of the assistantship opportunities I have been granted. I would like to thank Monique Eckerd for her time and effort in the animal facility assisting us in taking care of our zebrafish, as well as Sue Bauldry for her work in the generation of the antibody which was so crucial to my study.

I would like to thank all of my Biology graduate student peers for providing stress relief and a social outlet when work became overwhelming. My amazing committee members, Sue Edwards and Mary Connell, have not only been ready and willing to help with any obstacle I

have encountered, but have also provided deeper support, even when their plates were more full than mine, during my time here that I will remember for years, and for that I am so grateful. My primary advisor, Ted Zerucha, has been the most wonderful mentor that I could have asked for and has helped me grow and mature both personally and in my career. I cannot express how thankful I am for his guidance, and I know I would not be in the position that I am without his assistance, encouragement, and friendship throughout the last 5 years. Finally, to my parents and sister, thank you for the support and the love that you have shown me. I literally could not have gotten this far without you.

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INTRODUCTION

Early embryonic development is an immensely intricate process that depends on the strict regulation and expression patterns of numerous genes, which are long coding sequences of deoxyribonucleic acids (DNA) made up of the four nucleotides adenine (A), thymine (T) guanine (G), and cytosine (C). The process begins at fertilization of the egg by the sperm to form the zygote. This action initiates a cascade of critical events that all cooperate to eventually form a complex developed organism. The zygote immediately begins to undergo cleavage in order to rapidly increase the number of cells in the embryo. The cells produced during this stage soon undergo initial specification and eventual differentiation, creating the numerous types of cells that are critical to the adult organism. During this process, patterning of the body plan is also occurring along specific axes. In bilaterians, there are three main axes that develop: the anterior-posterior (AP) axis running from the head to the tail, the dorsal-ventral (DV) axis running from the topside to the underside, and the right-left (RL) axis running laterally from the right side of the body to the left. The events involved in the formation of these axes are strictly coordinated with each other as well as with the development of the organism's limbs, organs, and other internal systems. In order for all of these processes to successfully result in the formation of a complete adult organism, the genes involved in each must be under strict and constant regulation to prevent even minor malfunctions (Wolpert, 2007).

Due to obvious ethical conflicts, the study of developmental biology, especially as it pertains to medical applications in human embryonic development, has faced numerous

challenges until relatively recently. It was once a common belief that every organism possessed its unique physiology because of its unique set of genes, and that the complexity of these gene sets were directly correlated to the complexity of the organism (Carroll et al., 2008). This was the predominant belief as recently as the mid 1970s when the first conclusive evidence arose that the coding regions of the human genome were almost identical to the coding regions in the genome of the chimpanzee (King and Wilson, 1975). This prompted a deeper look into the coding regions of other organisms and provided extensive evidence towards the idea that non-coding regulatory regions are responsible for physiological diversity (Jacob, 1977; Prager and Wilson, 1975; Wilson et al., 1974). As more research delved into these areas, it became increasingly clear that the genes of most animals are highly similar in sequence and function and that phenotypic variations present among organisms are due in large part to evolution of the genes' regulatory regions (Carroll, 2005). This high gene sequence similarity provided additional support for the use of model organisms to study key developmental genes.

The zebrafish (*Danio rerio*), was first used for developmental studies by George Streisinger in the 1960s (Streisinger et al., 1981). His work was aided by the work of other developmental biologists such as Charles Kimmel, Christiane Nüsslein-Volhard, and Marc Fishman (Grunwald and Eisen, 2002). By the year 2000, a project to sequence the zebrafish genome had been initiated, and scientists around the world were gathering to discuss the potential of zebrafish as a model organism (Bamford et al., 2000; Grunwald and Eisen, 2002; Kimmel, 1989; Mullins et al., 1994).

Many of the attributes presented by these "founding fathers" demonstrate that the zebrafish lends numerous advantages to the study of embryonic development. Its genome,

although approximately half the size of the human genome, shares a high level of sequence identity with the human genome with respect to the coding sequences, making it a good model for studies wishing to provide insight into human development and genetics (Haffter et al., 1996; Warren et al., 2000). Female zebrafish can lay up to hundreds of eggs a week, allowing for relatively easy reproducibility of experiments, and the eggs are fertilized and develop completely externally, allowing for the simple visualization of the developmental process as early as the one-cell stage. The embryos are also optically transparent throughout their development, and their stages have been well documented, making the zebrafish a good organism to use in the study of developmental biology (Dooley and Zon, 2000; Grunwald and Eisen, 2002; Kimmel et al., 1995). One of the most interesting things about studying genetics in zebrafish is that the teleost lineage, of which the zebrafish is a member, underwent a whole genome duplication event after the split from tetrapods (Brunet et al., 2006). This duplication created two copies of many of the genes that most land animals such as humans possess, which, therefore, may allow scientists to tease out individual functions of multifunctional genes while also providing a model to study the effect of evolutionary conservation of specific sequences (Brunet et al., 2006; Jaillon et al., 2004).

In zebrafish development, following fertilization of the egg, rapid cell divisions result in hundreds of cells which then migrate around the yolk cell during epiboly. Approximately 6 hours past fertilization (hpf), the formation of the shield caused by cell convergence marks the first deviation from radial symmetry, and by 10 hpf the precursor to the AP body plan is clearly visible (Schier and Talbot, 2005; Solnica-Krezel, 2005; Wolpert, 2007). Development of this AP axis is crucial to the overall development of the embryo. As the embryo's development progresses, many characteristic patterning events occur along the AP

axis that must be critically regulated. Important mesodermal segments called somites begin to form along this axis that eventually give rise to the different vertebrae in the fish, while the formation of rhombomeres simultaneously occurs in the developing neural tube that precede the formation of different regions of the brain. In order for these necessary specifications to occur, each segment is characterized by a unique gene expression profile that is, in part, regulated by a set of genes called the *Hox* genes (Alexander et al., 2009; Burke et al., 1995; Grapin-Botton et al., 1995; Krumlauf, 1994). These genes, which are conserved across all vertebrates and have orthologous counterparts in invertebrates, carry out numerous different functions throughout embryonic development (Krumlauf, 1994).

The *Hox* genes were originally discovered in 1978 when a group of genes known as the bithorax complex was found to control body segmentation in the fruit fly *Drosophila melanogaster* (Lewis, 1978). These genes were located in a very specifically organized cluster and subsequently found to all contain the same 180 base pair sequence of DNA, named the homeobox, which codes for a 60 amino acid helix-turn-helix DNA-binding protein domain called the homeodomain (Gehring, 1993; McGinnis et al., 1984). It was soon discovered that this homeobox sequence was present in many genes in almost every multicellular organism, prompting researchers to group them into a homeobox gene superfamily. The *Hox* genes comprise a particular subset of this superfamily that plays a major role in patterning the AP axis during embryonic development (Lemons and McGinnis, 2006).

An interesting feature of the *Hox* genes is that they are always found in the same order relative to each other and always transcribed in the same direction (Ruddle et al., 1994). In addition to this unusual pattern, the *Hox* genes are also expressed in a temporal and

spatial manner that is consistent with their location. For instance, *Hox* genes in all organisms that are found towards the 3' end of the cluster are always expressed earlier and more anteriorly. Conversely, the genes found nearer to the 5' end of the cluster are expressed later during development and more posteriorly (Fig. 1). This feature is termed colinearity and is a unique function of the *Hox* gene clusters (Amores et al., 1998; Duboule, 1998; Lufkin, 1996; McGinnis and Krumlauf, 1992).

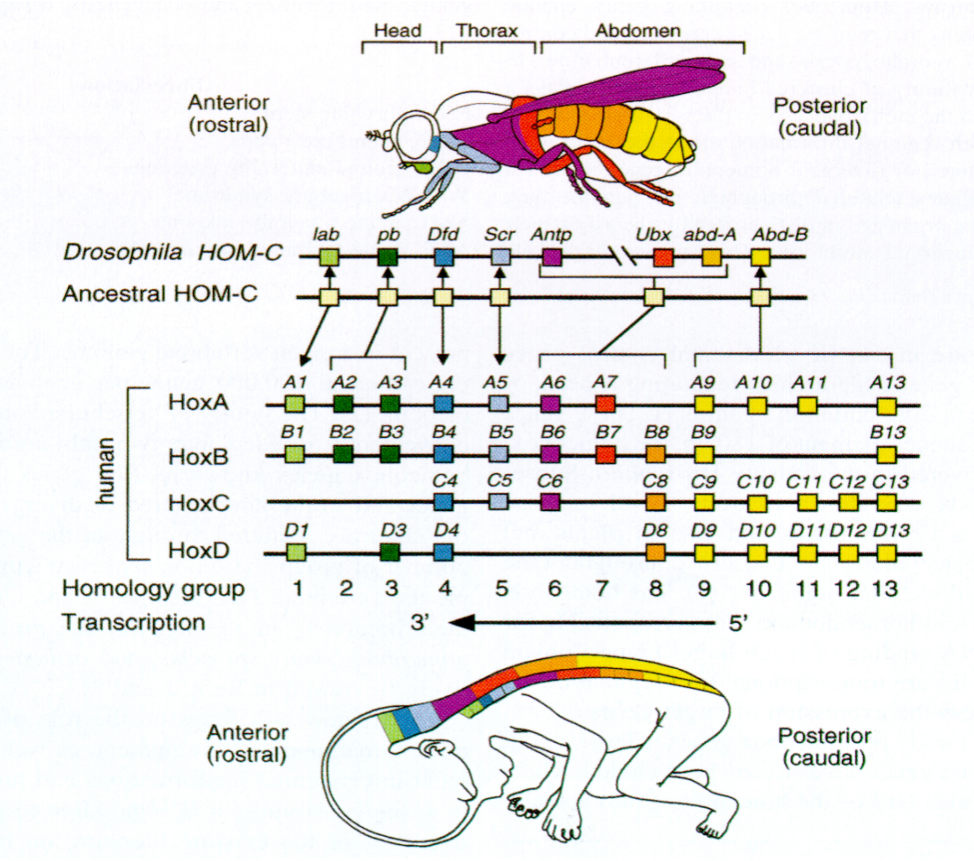

Fig. 1. Colinearity of *Hox* genes is conserved across various organisms. The *Hox* genes are present in conserved clusters in which the genes are organized linearly along the chromosome in the same manner in all organisms observed. Genes located toward the 3' end of the cluster are expressed earlier and more anteriorly than those located in the 5' end of the cluster. Figure taken from Mark et al., 1997.

While all cnidarians and bilaterians possess orthologs of the *Hox* genes that serve to pattern body development, the number of clusters an organism possesses seems directly correlated with its axial diversity. Invertebrates such as the fruit fly, for instance, possess only one full set of *Hox* genes while tetrapods have undergone duplication events resulting in four paralogous *Hox* clusters (Alexander et al., 2009; Amores et al., 1998; Holland et al., 1994). These four different clusters have been named *HoxA-D,* and individual genes are typically referred to by both their cluster letter and paralog number, lending names such as *Hoxa1* (Scott, 1992). Zebrafish, although a member of the vertebrate subphylum, have undergone the teleost specific genome duplication discussed previously. This duplication event initially resulted in two copies of the four tetrapod clusters which, after the suspected loss of one of these clusters, resulted in seven total zebrafish *Hox* clusters (Amores et al., 1998; Prince et al., 1998).

The homeobox region of the *Hox* genes, as mentioned above, codes for a helix-turn-helix DNA-binding motif. Thus, homeobox genes, including the *Hox* genes, code for proteins that act as transcription factors to preferentially regulate activation or repression of key genes during development by binding to sequences present within regulatory regions of their target genes (Botas, 1993; Dorn et al., 1994; Gehring, 1993). However, the sequence of DNA to which the homeodomain often binds is the short, four nucleotide sequence TAAT (Carroll, 1995; Ekker et al., 1994). This small binding site occurs randomly in the genome too often to allow these genes to function with their known specificity, which poses a problem because the main responsibility of homeodomain proteins is to strictly control the activity of other genes. A solution to this problem was proposed when it was shown that in many instances, the amino acid changes that differentiate the Hox proteins from each other

are within the domains that participate in protein-protein interactions, indicating that the Hox proteins have the potential to interact with numerous other proteins (Sharkey et al., 1997). Thus, in order to overcome this seeming binding site obstacle, Hox proteins work cooperatively with other homeodomain proteins, known as cofactors, to form three-dimensional complexes that significantly increase the area of DNA to which the proteins will bind. As there are both numerous Hox proteins as well as numerous cofactors that have different binding sites with which the Hox proteins can interact, the combinations of these proteins sufficiently overcome the lack of binding site specificity of the Hox homeodomain alone (Hoey and Levine, 1988; Hoey et al., 1988; Mann, 1995; Mann and Affolter, 1998).

The largest set of *Hox* cofactors that has been identified is the superclass of homeobox genes known as the TALE (three amino-acid loop extension) genes (Bertolino et al., 1995). These genes contain the helix-turn-helix homeodomain coding region but are characterized by having an extra three amino acids between helix 1 and 2 (Burglin, 1997; Gehring et al., 1994). The TALE superclass is further divided into two main subclasses, named PBC and MEIS, based on conserved motifs upstream of their respective homeodomains which cooperate in varying degrees with Hox proteins to increase both binding specificity and affinity to DNA (Affolter et al., 1999). The interactions between Hox proteins and members of the PBC protein family (such as Pbx) are typically accomplished both directly, via distinct differences in the Hox amino-terminal arms, and indirectly, via the combined DNA binding sites of the newly established protein complexes (Chang et al., 1996; Shen et al., 1996). Conversely, interactions between members of the PBC or Hox protein families and those of the MEIS family (Meis or Prep proteins) have been shown to occur in the presence or absence of the DNA, indicating that these can assist in increasing the binding

affinity of Hox/Pbx heterodimers or carry out purely protein-protein interactions for localization or stability purposes (Berthelsen et al., 1998; Berthelsen et al., 1999; Waskiewicz et al., 2001).

The *Meis* genes were first discovered in 1996 when the myeloid ecotropic leukemia virus integration site was found to interrupt a previously uncharacterized open reading frame containing a TALE-homeodomain coding region later named *Meis1*. Two more genes, *Meis2* and *Meis3*, were identified soon after via DNA-DNA hybridization studies using the *Meis1* homeobox region as a probe. Interestingly, a similar DNA-DNA hybridization experiment using a probe targeted towards the 3' untranslated region (UTR) of the *Meis1* gene did not pick up the same two novel genes. This showed that there were numerous novel genes sharing a highly conserved homeodomain region, but that differences in the UTRs of these genes may indicate different UTR-controlled expression profiles. Taken together, these data suggested the discovery of a new family of TALE superclass *Hox* cofactors (Moskow et al., 1995; Nakamura et al., 1996; Steelman et al., 1997).

After the initial *Meis* genes were identified in murine organisms, human orthologs were soon identified that presented a surprising amount of sequence identity across their respective counterparts (Steelman et al., 1997). This prompted a cascade of research that ultimately resulted in the identification of three *Meis* genes (1-3) in humans (Geerts et al., 1997) and other vertebrates such as chicken (*Gallus gallus*; Sanchez-Guardado et al., 2011a) and mouse (*Mus musculus*; Cecconi et al., 1997; Nakamura et al., 1996), an additional *Meis4* gene in zebrafish (*Danio rerio*; Biemar et al., 2001; Sagerstrom et al., 2001; Vlachakis et al., 2001; Waskiewicz et al., 2001; Zerucha and Prince, 2001), as well as a *Meis* ortholog in the fruit fly *Drosophila melanogaster* called *homothorax* (*hth*; Kurant et al., 1998; Pai et al.,

1998). In addition to the known *Meis* genes, *Meis1-3* in vertebrates have the ability to be alternatively spliced, adding to the diversity of their protein products (Huang et al., 2005; Maeda et al., 2001; Sanchez-Guardado et al., 2011b). Furthermore, it was discovered that the homeobox region of these *Meis* genes also shared a strong similarity to that of the *Pbx* genes, presenting the possibility of related protein functions (Moskow et al., 1995). Upon this revelation, the properties of the *Meis* genes were more thoroughly characterized in order to tease out exactly what function they were playing that resulted in such unique features.

The *Meis* genes code for homeodomain-containing proteins that have the ability to either bind DNA or interact with other proteins via two distinct domains. The homedomain region of the Meis1 protein was found to bind to a highly conserved 6-nucleotide sequence TGACAG (Chang et al., 1997; Moskow et al., 1995; Shen et al., 1997). This was an unexpected finding because, although the homeobox sequence of the *Meis* genes is remarkably similar to that of the *Pbx* genes, the latter differs in that its protein product follows the canonical homeodomain DNA-binding pattern, recognizing the tetrameric TAAT core sequence (Chang et al., 1997; Lawrence and Largman, 1992). Also, whereas the majority of homeobox genes have few introns, in most organisms the *Meis* genes contain an unusually high 10 to 11 introns (Irimia et al., 2011). In addition to the ability of the Meis proteins to bind their target hexameric sequence alone, they have also been shown to cooperatively bind DNA in conjunction with other homedomain proteins such as Hox or Pbx. This occurs via protein-protein interactions of non-homeodomain regions of these proteins that will be discussed later. This phenomenon occurs via binding of the newly formed protein complexes to DNA binding sites comprising each individual protein's full or partial recognition sequences lying adjacent to one another (Chang et al., 1997; Shanmugam et al.,

1999; Shen et al., 1997; Shen et al., 1999; Swift et al., 1998b; Waskiewicz et al., 2001). The formation of these three-dimensional complexes is the mechanism by which the Meis proteins function to increase binding specificity and therefore target gene regulation (Mann and Affolter, 1998; Fig. 2).

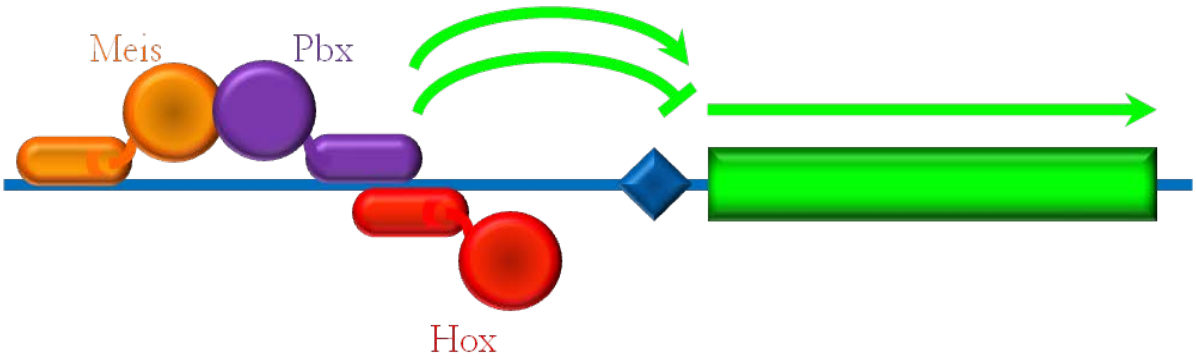

Fig. 2. Trimeric complex of Meis, Pbx, and Hox proteins increases binding specificity and target gene regulation. Each protein binds its individual recognition sequence within the enhancer region of the target gene. Whereas the individual DNA (deoxyribonucleic acid)-binding sites are short and non-specific, the combination of the binding sites of the proteins involved in the complex create a longer and more precise regulatory region, increasing the likelihood of controlled regulation of the target gene during development. Image adapted from Berthelsen et al., 1998.

While the Meis proteins interact with their DNA-binding sites via the homeodomain regions, their interactions with other proteins occur using other domains within the protein (Burglin, 1998; Shen et al., 1997). In contrast with genes from the PBC family that interact with the more 3', anteriorly expressed *Hox* genes, the *Meis* genes primarily interact with the more posteriorly expressed *Hox* genes located towards the 5' end of the cluster (Mann and

Chan, 1996; Shen et al., 1997). This interaction with the posterior Hox proteins takes place between the N-terminal region of the Hox proteins (amino acids 1-61 in Hoxa9, for instance) and the C-terminal region of the Meis proteins (Shanmugam et al., 1999; Shen et al., 1997; Williams et al., 2005). In contrast, Meis proteins interact with PBC proteins via their respective amino terminal ends to form DNA-binding heterodimers (Burglin, 1998; Chang et al., 1997; Knoepfler et al., 1997). The PBC-interacting domain on the N-terminal end of the Meis proteins is conserved with their fruit fly ortholog *hth* and is therefore termed the Hth domain (Hyman-Walsh et al., 2010). Through the utilization of their numerous domains, the Meis proteins can both successfully achieve the formation of dimeric or trimeric complexes as well as functionally bind DNA to carry out their cofactor responsibilities.

Interestingly, Meis proteins have been shown to form functional complexes with other homeodomain proteins even in the absence of DNA, indicating additional functions (Waskiewicz et al., 2001). For instance, Meis proteins have been shown in both the fruit fly and zebrafish to interact *in vitro* with Pbx proteins without DNA being present to facilitate binding. This interaction localizes the Pbx proteins within the nucleus, where they can carry out their transcription factor functions, rather than being transported into the cytoplasm of a cell where they are frequently found (Berthelsen et al., 1999; Mann and Abu-Shaar, 1996; Waskiewicz et al., 2001). In this way, the Meis family also plays a role outside of its own DNA-binding functions in regulating the PBC family's transcription factor activity. Therefore, this also indirectly regulates the ability of the Hox proteins to direct embryonic gene expression by affecting their capability to utilize the extra DNA-binding specificity of the PBC proteins. Taken together, these data began to suggest roles for the *Meis* genes outside of cofactor activity. Upon further observation, the individual *Meis* genes serve a

myriad of roles in the developing embryo, not all of which are related to their functions as homeodomain proteins.

The first of the Meis family to be discovered, *Meis1*, was found when it was shown that the integration of a myeloid leukemia virus interrupted its normal protein sequence. This caused erratic expression and behavior of *Meis1* and resulted in the formation of leukemia, which is consistent with the misregulation of other homeobox genes due to their tendency to play a significant role in hematopoietic cell differentiation and proliferation (Hatano et al., 1991; Lawrence and Largman, 1992; Moskow et al., 1995; Perkins et al., 1990). *Meis1* has been shown to be highly expressed early in development in numerous organisms including the zebrafish, chicken, and the African clawed frog *Xenopus* with an expression pattern that spans throughout the mesoderm and somites early on but becomes largely anteriorly localized as embryonic development continues (Coy and Borycki, 2010; Maeda et al., 2002; Steelman et al., 1997; Waskiewicz et al., 2001). This large expression profile allows *Meis1* to carry out a number of various functions throughout development.

In addition to its homeodomain protein role in which it cooperates with Hox and Pbx proteins to set up the AP axis, *Meis1* has been found to play a key role in limb development in vertebrates. During early limb development, *Meis1* expression is up-regulated by retinoic acid, thereby inhibiting the distal patterning of the limb and allowing the limb to extend proximally. Its reduction in expression later in development allows distal patterning to take over, a process which is thought to be evolutionarily important in the distal growth of limbs (Mercader et al., 1999; Mercader et al., 2000). *Meis1* is also implicated in the development of the pancreas by regulating transcription of *Pax6* (Zhang et al., 2006). Its protein product is found to be expressed in adult bone marrow and fetal liver hematopoietic stem cells which,

taken together with its involvement in myeloid leukemia, indicates a prominent role in hematopoiesis. This is further supported by the overexpression of *Meis1* in numerous other types of leukemia in humans as well as the severe lack of development of the vasculature and hematopoietic system in mice in *Meis1* mutants (Afonja et al., 2000; Azcoitia et al., 2005; Imamura et al., 2002; Pineault et al., 2002). Furthermore, in zebrafish, *meis1* (a product of the teleost-specific genome duplication, specifically the duplication of the *Meis1* gene), plays a distinct role in the development of the endothelial intersegmental vessels as well as artery differentiation (Minehata et al., 2008). In early eye development in zebrafish, *meis1.1* functions by maintaining cell proliferation via regulation of two common cell-cycle activators, *cyclin D1* and *c-myc*, indicating novel roles for *meis1* in the cell cycle, and has also been implicated in the differentiation and specification of retinal stem cells (Bessa et al., 2008; Erickson et al., 2010). Similar studies have provided evidence for these *Meis1* functions in mouse and chicken retinal development (Heine et al., 2008).

The Meis1 protein also plays a crucial role in hindbrain segmentation as an indirect result of its interactions with Pbx proteins. It has been demonstrated in zebrafish that after mutating the *meis1.1* gene, the resulting phenotype is identical to that of the *lazarus* (*lzr*) mutant which results from disruption of a zebrafish *pbx* homolog with which *meis1.1* interacts (Waskiewicz et al., 2001). The lack of *meis1.1* causes a reduction in the expression of key hindbrain patterning genes such as *krox20* and various *hox* members (Waskiewicz et al., 2001). Interestingly, the zebrafish *meis1.2* seems to have been lost, resulting in *meis1.1* being largely referred to in the literature simply as *meis1* (Irimia et al., 2011; Minehata et al., 2008).

The second Meis family gene, *Meis2*, also functions in a variety of ways outside of its Hox-cofactor responsibilities. It was first identified through a DNA-hybridization experiment using the *Meis1* homeobox as a probe and initially named *Meis-related gene 1* (*Mrg1*) (Nakamura et al., 1996; Steelman et al., 1997). The name was later changed to *Meis2* when it was shown that its homeodomain region was identical to that of *Meis1* (Oulad-Abdelghani et al., 1997). Its ability to form trimeric complexes with proteins from the Hox and Pbx families is highly similar to that of *Meis1* (Chang et al., 1997), but *Meis2* also maintains distinct functions and expression patterns. Early in development, *Meis2* is expressed in the developing neural tube, somitic mesoderm, cranial and dorsal root ganglia, forebrain, midbrain, hindbrain, and developing limb buds (Cecconi et al., 1997; Oulad-Abdelghani et al., 1997; Toresson et al., 2000). In humans and mice, the *Meis2* gene has been shown to have at least 5 isoforms, each of which possesses the full conserved homeodomain sequence but differs in various other regions (Oulad-Abdelghani et al., 1997; Yang et al., 2000).

Meis2 expression largely overlaps with *Meis1* in the developing limb bud in vertebrates, performing a seemingly redundant function in proximal specification to promote limb outgrowth. Similarly, the retraction of *Meis2* from the distal region of the developing limb bud allows distalization of the limb, indicating that this gene, like *Meis1*, assists in the organization of the proximodistal axis (Capdevila et al., 1999; Mercader et al., 2005). Another function of *Meis2* similar to that of *Meis1* is its role in pancreatic function. In contrast with *Meis1*, however, the protein product of one of the *Meis2* isoforms, *Meis2b*, has been shown to form a complex with *Pdx1*, a homeobox gene involved in the development of organs derived from endoderm, specifically involved in the development of the exocrine and

endocrine functions of the developing pancreas (Brooke et al., 1998; Stoffers et al., 1997). This complex serves to activate an enhancer for a key gene *ELA1* in acinar cells, which participate in the exocrine function of the pancreas by secreting digestive enzymes (Liu et al., 2001; Swift et al., 1998a).

Meis2 also plays various roles in brain and eye development. In mice, *Meis2* is heavily expressed in the developing neural tube, branchial arches, and somitic mesoderm, as well as the layer of endoderm covering the somitic mesoderm (Cecconi et al., 1997; Toresson et al., 2000). In monkeys, *Meis2* is expressed in the developing striatum, indicating a primary role in the developing forebrain (Takahashi et al., 2008). In the developing retina of humans, mice, and the medaka fish (*Oryzias latipes*), the function of *Meis2* has been implicated via its documented regulation of *Pax6* in specific types of amacrine cells, which mediate synapse information in the developing eyes (Bumsted-O'Brien et al., 2007; Conte et al., 2010; Wassle, 2004). This activity has also been recently confirmed in adult retinae of humans, mice, chickens, rats, and other vertebrate organisms, indicating for the first time a concrete function of *Meis2* in an adult organism (Bumsted-O'Brien et al., 2007). Preliminary evidence is also currently emerging of *Meis2* roles novel to the *Meis* family of genes, such as involvement in the formation of cleft palate and congenital heart disease, both of which have been shown to develop as a result of a *Meis2* exon deletion (Crowley et al., 2010).

In zebrafish, both *meis2.1* and *meis2.2* have been preserved and play distinct roles in development. Transcripts of the *meis2.1* gene are expressed throughout development, beginning at embryonic gastrulation (approximately 60% epiboly) and quickly expanding throughout the embryo as epiboly progresses. Later *meis2.1* is restricted to the developing forebrain and the area covering the posterior midbrain and anterior hindbrain, as well as in

the spinal cord, indicating a role in the central nervous system (Biemar et al., 2001; Zerucha and Prince, 2001). *Meis2.2* expression has been described in a similar pattern, being present in the branchial arches, eye fields, forebrain, and hindbrain (Waskiewicz et al., 2001).

The last of the Meis family of genes that has been extensively researched in vertebrates is *Meis3*. Like *Meis1* and *Meis2*, *Meis3* is first expressed during the gastrula stage of early embryonic development and is expressed primarily in the caudal hindbrain primordium (Salzberg et al., 1999; Vlachakis et al., 2001; Vlachakis et al., 2000). It has been shown in the African frog *Xenopus* that *Meis3* plays a very significant role in hindbrain patterning during development. Its protein product forms trimeric complexes with Hoxb1b and Pbx4 to induce the expression of genes involved in hindbrain development such as *Hoxa1*. When *Meis3* is expressed ectopically in the rostral region of the brain (forebrain and midbrain), it causes caudalization, or the transformation of the forebrain and midbrain to hindbrain cell fates, and its expression is sufficient to promote differentiation of hindbrain cell fates (Choe et al., 2002; Salzberg et al., 1999; Vlachakis et al., 2000; Vlachakis et al., 2001). This control over hindbrain patterning, while not fully understood, has been shown to be a result of the coordination by *Meis3* of retinoic acid activity, Wnt signaling pathways, and FGF/MAP Kinase pathways. In cooperation with the Wnt signaling pathway, *Meis3* also plays a role in organizing the development of the caudal neural plate as well as the induction of primary neurons and the neural crest (Aamar and Frank, 2004; Dibner et al., 2004; Elkouby et al., 2010; Gutkovich et al., 2010).

Meis3 plays a unique role in cell survival, as evidenced by its role in pancreatic islet cells and β-cells. Research has shown that while all three *Meis* genes described thus far have been found in the pancreatic β-cells, *Meis3* has significantly higher levels of expression, and

its absence has been shown to result in increased cell death. This activity is accomplished via regulation of *Pdk1*, a gene involved in cell survival in apoptosis regulation. This role in survival is carried into the pancreatic primary islets which, along with the role of *Meis3* in the β-cells, promotes healthy pancreatic functions and may prevent the underlying causes of diabetes. Interestingly, *Meis3* and *Pdk1* are also found in ovarian cancer cells, and when *Meis3* is silenced, the cancer cells undergo a cell death event (Liu et al., 2010). This is a novel function for the *Meis3* gene, and is currently being researched as a potential target for therapies for these diseases.

In zebrafish, *meis3.1* is the only homolog that has been identified, indicating that like *meis1.2*, *meis3.2* has not been evolutionarily conserved but has instead been lost since the teleost genome duplication event. Its main role that has been described to date is that of hindbrain patterning and its expression coincides with *Meis3* expression in the frog as described above, particularly in the early hindbrain primordium (Choe et al., 2002; Vlachakis et al., 2001; Waskiewicz et al., 2001). Two novel *meis* genes have also been described in zebrafish, *meis4.1* and *meis4.2*, but apart from confirming the presence of the homeobox sequence within these genes, very little work has been done delving into their functions, and relatively little information is available on them (Waskiewicz et al., 2001).

Interestingly, while functions for all of the *Meis* homologs in numerous organisms have been studied extensively, our lab has identified a novel gene downstream of *Meis2* for which no previous data exist. This gene, labeled *zgc:154061* in the Zebrafish Model Organism Database ZFIN, and which we have temporarily named *Meis2 linked gene* (*M2lg*), is present in every vertebrate for which genome data is publicly available. It is always located directly downstream of *Meis2* and is always transcribed in the opposite direction

(Carpenter, 2010; Graham, 2009). In zebrafish, *m2lg* is only located downstream of *meis2.2* and not *meis2.1* (Carpenter, 2010). Within its introns, it contains putative regulatory elements that are also located downstream of *Meis2* (only one putative regulatory element is present in zebrafish, again downstream of *meis2.2*) and conserved across almost all organisms with available genome data (Nelson, 2011). In light of recent research documenting the existence of genomic regulatory blocks (GRBs), we hypothesize that *M2lg* likely belongs to a newly described set of genes called "bystander genes" (Kikuta et al., 2007b).

GRBs have recently been discovered as increasing numbers of highly conserved non-coding elements (HCNEs; small regions of non-coding DNA that convey regulatory control over their target genes) have been documented in various vertebrate organisms (Kikuta et al., 2007b; Woolfe et al., 2005). A GRB typically consists of a stretch of genes that is present in the same order across numerous organisms, indicating a positive evolutionary pressure on the region. One of the defining factors of a GRB is the conserved presence of numerous HCNEs controlling expression of one of the genes within the block. The accepted model of function for the HCNEs is that each element individually controls one aspect of the target gene's expression and function, and that the entire array of HCNEs for any target gene is sufficient to account for all of the functions the target gene maintains (Engstrom et al., 2007; Kimura-Yoshida et al., 2004; Woolfe et al., 2005). These HCNEs are usually present within and around the introns of the surrounding gene(s), providing a potential theory for the high degree of syntenic conservation within a GRB (Engstrom et al., 2007; Gomez-Skarmeta et al., 2006; Kikuta et al., 2007b). As the HCNEs are responsible for directing target gene expression in development via binding of sequence-specific transcription factors (Hardison,

2000; Woolfe et al., 2005), it is proposed that the high level of pressure on the HCNEs to maintain their sequences and positions is also transferred to the genes encompassing them (Kikuta et al., 2007b).

It has been thought previously that the high degree of conservation present within these regulatory blocks was a direct result of all genes involved being under the control of the HCNEs present within the sequence (Carvajal et al., 2001; Goode et al., 2005; Spitz et al., 2003). More recently, however, evidence has surfaced indicating that these HCNEs have only one target gene, and that the evolutionary pressure is on the HCNEs to maintain synteny, sweeping along the "bystander gene(s)" with them. Interestingly, in many cases, the bystander gene(s) expression pattern is similar to the target gene pattern but is much less specific and in many instances is completely functionally unrelated to the target gene (Kikuta et al., 2007a; Kikuta et al., 2007b).

The control of developmental regulatory genes is often associated with significantly large, conserved GRBs termed Ultra-Conserved Elements (Bejerano et al., 2004). These are defined as regions of DNA at least 200 base pairs in length that map with 100% identity between the genomes of the human, mouse, and rat. Bejerano et al. found 481 of these segments within the genome, most of which also shared significant (>90%) sequence similarity with the dog and chicken genomes, further strengthening the evidence for their syntenic relationships. More than 80% of these sequences have been shown to be associated with genes that are active during embryonic development, with many of them being important embryonic regulatory genes such as the *Hoxd* cluster and *Pbx3* (Sandelin et al., 2004; Spitz et al., 2003). The GRBs described by Kikuta et al. (2007a) have also been located around developmentally important regulatory genes such as *FGF8* (the human

growth factor), *Pax6* (involved in central nervous system and retinal development), *Rax* (essential for retinal development), and S*hh* (involved in a multitude of essential roles in a developing organism; Goode et al., 2005; Jeong et al., 2006; Kikuta et al., 2007b). This evidence, along with the linkage of *M2lg* and *Meis2* in every organism we have observed, provides support for our hypothesis that the *Meis2* gene may be part of one of these regulatory blocks.

One of the concerns with confirming the evolutionary maintenance of the bystander genes is that of comparing organisms that have diverged at various times. Many times, comparing the human genome with that of the mouse or rat can return numerous false positives simply due to the lack of evolutionary divergence (Nobrega and Pennacchio, 2004). Work done in the fruit fly and several other insects has provided information not only about the evolutionary divergence of vertebrates from invertebrates, but also concerning the presence and necessity of GRBs in metazoan genomes (Engstrom et al., 2007). Additionally, the teleost fish also present a very interesting model organism with which to study GRBs. Their level of divergence from humans and other mammals often gives insight into ancient regulatory elements that hold key functions for expression of important developmental genes (Aparicio et al., 1995; Goode et al., 2003; Nobrega et al., 2003; Zerucha et al., 2000). This aspect of genome divergence also provides information about the more recent evolution of these genes' regulation, providing insight into varying phenotypic morphology due to differential regulation (Boffelli et al., 2004). Another interesting aspect of using teleost fish to study GRBs is that, due to the teleost-specific genome duplication, teleost fish at one point presumably contained two copies of the tetrapod entire genome (Jaillon et al., 2004; Meyer and Van de Peer, 2005; Woods et al., 2000). In duplication events such as these, wherein

two copies of the same gene, along with its associated HCNEs, arise, both genes are not strictly required for the function of the organism. In this instance, one or both are no longer under strict evolutionary pressure (Force et al., 1999). When this happens, Force et al. (1999) propose that the genes are subject to mutational events.

In the case of a duplication event, copies of coding or HCNE sequences can undergo one of three events (Force et al., 1999). The first of these events, nonfunctionalization, occurs when one of the genes or HCNE copies is mutated in a way that prevents it from functioning. In this case, evolutionary pressure is immediately placed on the remaining gene and its associated HCNEs, allowing the mutated sequences to become lost over time while preserving the other copy in a more or less ancestral state. In subfunctionalization, when the pressure is removed from both HCNE sequences because of their initial absolute redundancy, random mutations occur at both loci that render complementary components of them individually nonfunctional. Once this occurs, pressure preserves only the HCNE that maintains its ancestral function. This random loss of HCNE sequences results in two copies of the gene that each have separate functions, but together perform all the functions of the original gene. The final event, neofunctionalization, is what Force et al. (1999) propose as the driving force of evolution. In this situation, a mutation occurs in one of the HCNEs that, rather than making it nonfunctional, instead introduces a novel function. While one HCNE carries on the gene's traditional function, the mutated HCNE confers a new function onto the gene. This model, termed the Duplication-Degeneration-Complementation model, is the proposed mechanism behind the evolution of body morphology and increasing organism complexity (Force et al., 1999; Fig. 3).

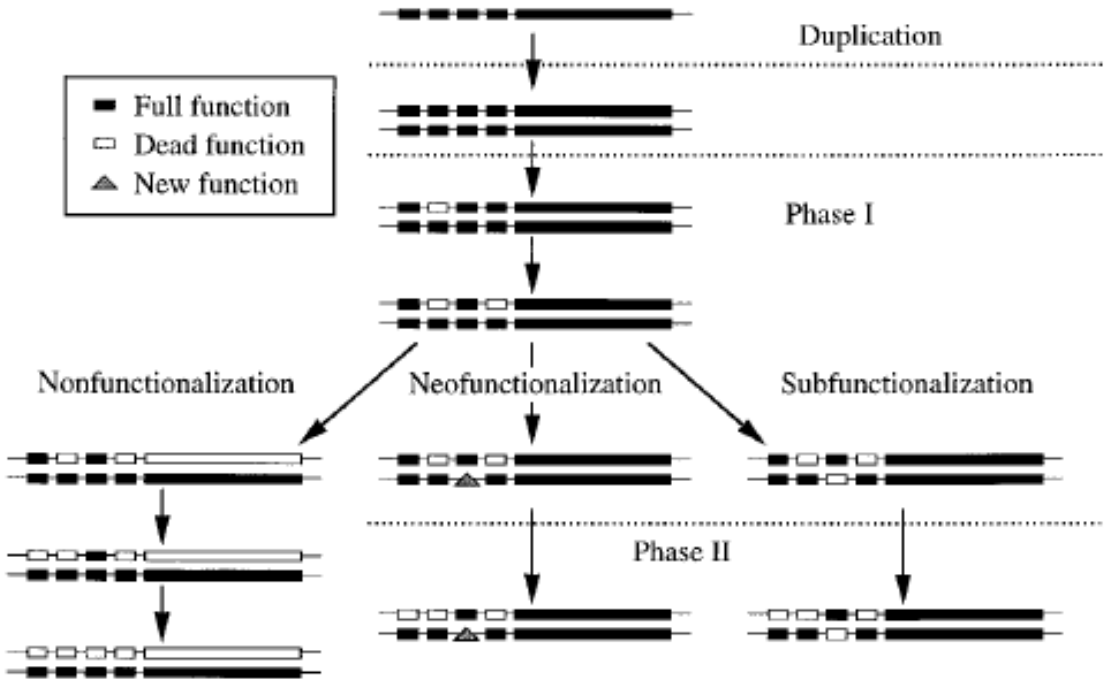

Fig. 3: The Duplication-Degeneration-Complementation model. This model, taken from Force et al., 1999, describes the three possible fates of gene copies and their respective highly conserved non-coding elements (HCNEs) following a duplication event. In nonfunctionalization, the duplicated gene quickly amasses mutations that remove its ability to carry out its function. Following this event, the gene and its associated HCNEs are no longer under any pressure due to the lack of function and they are randomly mutated over time. The middle option, neofunctionalization, occurs when one HCNE accrues a random mutation that translates into a novel function for the gene when the HCNE is activated. This is the most likely process through which body morphology evolution occurs. The last option, subfunctionalization, is the product of the random loss of the HCNEs as a result of relaxed evolutionary pressure. Whichever HCNE is mutated first is lost and pressure is once again applied to its duplicate to maintain its sequence. This results in two copies of the gene that operate individually but together encompass the entire original gene function.

Because of the genome duplication in teleost fish, it is likely that nonfunctionalization, neofunctionalization, or subfunctionalization has since occurred in many of the duplicated genes. This can be explored when looking at bystander genes in GRBs. In many cases, one or more genes in a GRB have been lost due to the temporary relaxed evolutionary pressure that occurred following the genome duplication. This gives insight into which of the genes is the target gene of the HCNEs present within the GRB, which is a critical step in determining the regulatory function of any given block (Force et al., 1999; Kikuta et al., 2007a; Kikuta et al., 2007b).

It has been shown previously within our lab that the *m2lg* mRNA (messenger ribonucleic acid) expression overlaps significantly with that of *meis2.2* in zebrafish, but with a much less localized expression pattern (Carpenter, 2010). Additionally, *m2lg*, along with the putative regulatory element we have identified in zebrafish, are only located downstream of *meis2.2* and have seemingly been lost next to *meis2.1*, potentially as a result of a nonfunctionalization following the teleost genome duplication (Nelson, 2011). Therefore, we propose that the putative regulatory element, as well as *M2lg* located downstream of the *Meis2* gene, make up a genomic regulatory block for *Meis2*, *meis2.2* in zebrafish, and that *M2lg* is a potential bystander gene held in place by the evolutionary pressure on the putative regulatory elements. The work reported in this thesis documents the steps taken towards characterizing the protein product of *m2lg* in zebrafish as we work towards functional studies of this novel gene.

MATERIALS AND METHODS

Zebrafish Husbandry

Zebrafish used for laboratory purposes were housed in a Marine Biotech Z-mod (Aquatic Habitats, Apopka, FL) closed system and maintained as described in *The Zebrafish Book: A guide for the laboratory use of zebrafish* (*Danio rerio*) (Westerfield, 1993). Fish of the wild-type lines AB, AB*, and TU (Zebrafish International Resource Center) as well as of non-genetically controlled lines (Carolina Biological, Burlington, NC) were maintained in the system at a constant water temperature of 27°C on a 14 hour light/10 hour dark cycle. The water quality of the system was monitored daily such that the pH of the water was kept above 7.0 and the conductivity of the water was maintained between 550 and 600 milliSiemens per meter (mS/m). Adult fish were housed in individual 1L aquaria housing 7 fish maximum per aquarium.

In order to maintain each genetic line, fish were bred and the embryos were raised to adulthood. Breeding of fish was accomplished by separating the males and females of the same genetic line by a plastic divider within a specialized breeding chamber (Aquatic Habitats) in a 1L aquarium overnight. Because zebrafish are inherently social breeders, multiple males and females were housed in an individual breeding tank holding up to a maximum of 7 total fish, typically with males outnumbering females. The plastic dividers were removed within an hour of the onset of the light cycle to allow breeding to occur, during which the females would release their eggs and the males would subsequently fertilize them. After the resulting embryos fell through the mesh bottom of the breeding chambers,

the bred fish were placed in a separate 1L aquarium and replaced into the system. The embryos were then harvested by filtering with fine mesh and thoroughly rinsed with Reverse Osmosis (RO) water. Embryos were placed in small glass bowls and raised for 5 days in 1X (working concentration) Danieau buffer [58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5 mM HEPES pH 7.6] at 27°Celsius (C).

After the initial 5 days, embryos were transferred to a 1L aquarium filled approximately halfway with 1X Danieau buffer to allow room for the larvae to swim and grow. At this point, fine particulate dry food (Zeigler, Gardners, PA) feedings began twice daily. To increase survival rates of the larvae, 60 to 70% of the 1X Danieau buffer was replaced three times weekly to remove old food as well as replenish dissolved oxygen and renew nitrogenous waste neutralization capabilities.

At 20 days past fertilization (dpf), the larvae were placed under a slow drip of water in the Marine Biotech system, slowly equilibrating the fish to system water. Feedings continued twice daily, with the particulate size of food increasing as the fish increased in size (ZM-100, ZM-200, ZM-300, ZM-400; Zeigler). When the larvae were large enough to safely digest ZM-200, approximately 6 to 8 weeks past fertilization, they were also fed 2 day old live brine shrimp (INVE Aquaculture, Salt Lake City, UT) once daily in addition to the dry food regime. As the fish increased in size, they were separated into multiple tanks based on body size to encourage steady growth rates and prevent overpopulation. The fish reached adult size and reproductive maturity approximately 3 months after fertilization, at which point they were housed in 1L aquaria containing 5 to 7 individuals as described above and fed twice daily with Zeigler Adult Zebrafish Complete Diet while maintaining the once daily feedings of 2 day old live brine shrimp.

zgc:154601 Identification

A putative open reading frame *zgc:154601* was identified approximately 10.9 Kilobases (Kb) downstream of the zebrafish *meis2.2* gene by Dr. Ted Zerucha. We have temporarily named this gene *meis2 linked gene* (*m2lg*). The open reading frame comprises approximately 1914 base pairs (bp), and codes for a predicted protein sequence that is 300 amino acids (aa) in length and approximately 34 kD in size (Carpenter, 2010; Graham, 2009).

Antibody Generation

A polyclonal antibody was generated against a small peptide portion of the m2lg aa sequence. A multiple sequence alignment of the full predicted protein sequence of M2lg in zebrafish, human, mouse, and chicken was constructed and sent to Biosnythesis, Inc. (Lewisville, TX). The company determined which portion of the protein sequence would be most likely to elicit an immune response in an animal that would produce an antibody, and constructed that sequence into a peptide. The peptide was given to Appalachian State University's Spring 2010 Immunology laboratory under the charge of Dr. Sue Bauldry. Dr. Bauldry injected the peptide at a concentration of 1.01 milligrams per milliliter (mg/mL) into a naïve New Zealand white rabbit (RSI Biotechnology, Mocksville, NC) a total of 5 times at 0 weeks (wks), 3 wks, 6 wks, 9 wks, and 11 wks to elicit a secondary immune response and induce the formation of IgG antibodies. At 12 wks, the rabbit was exsanguinated using xylazine and ketamine as anesthetic agents. The serum obtained was allowed to clot for 16 hours overnight and was then centrifuged to pellet the clotting factors. The serum was obtained as supernatant after centrifugation, aliquoted into 1 milliliter (mL) aliquots and stored at -80°C.

Antibody Purification

IgG antibodies were purified from total rabbit serum using a Nunc™ ProPur™ Mini Protein Purification kit for Protein A (Thermo Scientific, Rockwood, TN) according to the manufacturer's instructions. Briefly, 1 mL total rabbit serum was filtered through a 0.2 micrometer (µm) syringe filter to remove solid particulates and passed through a spin column that binds IgG antibodies. The column was washed three times to remove non-immunoglobulin components, and the IgG was eluted into two fresh 1.5 mL microcentrifuge tubes to ensure that the maximum amount of IgG was recovered. The spin column was regenerated according to the manufacturer's instructions and stored at 4°C for reuse in future purifications.

Each time an aliquot of antibody was purified using the ProPur™ system, purification was confirmed via SDS-Page gel analysis. Samples of the filtered serum, diluted serum, wash steps, and eluted antibody were prepared by adding 15 µL of each sample to 5 µL 3X Laemmli loading dye [240 mM Tris pH 6.8, 6% sodium dodecyl sulfate (SDS), 30% Glycerol, 0.16% β-mercaptoethanol, 0.006% w/v (weight/volume) Bromophenol Blue, (Laemmli, 1970)]. Samples were heated at 70°C for 5 minutes to denature then immediately placed on ice to maintain denatured conformation. Samples were loaded into an SDS-PAGE gel consisting of a 5% stacking gel [70% RO H_2O, 16.5% acrylamide: Bis 29:1, 125 mM Tris pH 6.8, 0.1% w/v SDS, 0.1% w/v ammonium persulfate (APS), 0.1% *N,N,N'N'* tetramethylethylenediamine (TEMED)] and 12% resolving gel [32% RO H_2O, 40% 30% acrylamide:Bis 29:1, 390 mM Tris pH 8.8, 0.1% w/v SDS, 0.1% w/v APS, 0.04% TEMED]. Protein gel was immersed in 1X Tris-Glycine Running Buffer [25 mM Tris Base, 192 mM Glycine, 3 mM SDS] and electrophoresis was performed for 90 minutes at 250 volts.

Following gel electrophoresis, the stacking gel was removed using a razor blade and the resolving gel was stained to allow visualization of the protein bands. The gel was washed 3 times by submerging it in approximately 100 mL fresh RO H_2O, microwaving for 30 seconds, then incubating at room temperature with constant shaking for 5 minutes. This was followed by submerging the gel in 100 mL Coomassie Stain [70 mg Brilliant Coomassie Blue, 1L RO H_2O, 3 mL glacial hydrochloric acid (HCl)], microwaving for 10 seconds, and incubating at room temperature with constant shaking overnight. The gel was then destained by placing it in RO H_2O and incubating at room temperature until bands were clearly visible.

Following purification, eluted antibody concentration was analyzed via Bradford assay (Pierce Biotechnology BCA Protein Assay Kit, Thermo Scientific, Rockwood, TN) using the provided Bovine Serum Albumin (BSA) as a standard according to the manufacturer's protocol. BSA concentrations of 2000 µg/µL, 1500 µg/µL, 1000 µg/µL, 750 µg/µL, 500 µg/µL, 250 µg/µL, 125 µg/µL, 25 µg/µL, and 0 µg/µL diluted in RO H_2O were analyzed in triplicate using a plate reader (Soft Max Pro 5.2, Molecular Devices LLC, Sunnyvale, CA) to create a standard curve. Antibody eluates were also analyzed in triplicate both undiluted and diluted 1:10 and 1:25 in RO H_2O. Results were compared to the BSA standard curve and adjusted for dilution, then averaged to determine purified antibody concentration. Purified antibody was aliquotted into 1.5 mL microcentrifuge tubes in 45 µL aliquots and stored in 5% glycerol at -20°C.

Western Blot

Western blots were performed on total protein samples from embryos at various stages. Zebrafish breeding tanks were set up as described previously and embryos were obtained and allowed to grow in 1X Danieau buffer at 27°C. Embryos were harvested at 2

hpf, 4 hpf, 6 hpf, 8 hpf, and 12 hpf using a zebrafish embryo staging guide (Kimmel et al., 1995). Once 400-600 embryos of a stage were harvested, embryos were placed into 1.5 mL microcentrifuge tubes and flash frozen in liquid nitrogen. Frozen embryos were stored at -80°C to preserve all proteins present at the time of harvest.

Once enough embryos were obtained at each stage, frozen embryos were placed into a solution containing 1 mL protein homogenization buffer [250 mM sucrose, 30 mM Tris, 1 mM EDTA, pH 7.8, 5 µL proteinase inhibitor cocktail (PIC), and 10 µL phenylmethylsulfonyl fluoride (PMSF)]. Samples were homogenized using the Tissue Tearor™ Homogenizer (Cole-Parmer, Vernon Hills, IL), cleaning the homogenizer with double-rinses of RO H_2O between each sample. Cleared liquid from each sample was transferred to clean 1.5 mL microcentrifuge tubes and centrifuged at maximum speed for 10 minutes at 4°C. Supernatant for each sample containing the homogenized protein samples was transferred to fresh 1.5 mL microcentrifuge tubes and protein concentration determined using the Pierce Biotechnology BCA Protein Assay as described above.

Samples were loaded into 4-15% gradient polyacrylamide Mini PROTEAN® TGX™ pre-cast gels (Bio-Rad, Hercules, CA) at a total protein volume of 35 µg of sample for each stage. The samples were electrophoresed in 1X Tris -Glycine Running Buffer using a Mini-PROTEAN electrophoresis cell (Bio-Rad, Hercules, CA) at 200 volts for 1 hour to allow for protein separation into bands based on protein size. During electrophoresis, the cell was submerged in ice to prevent the buffer from overheating and altering the tracking properties of the protein within the gel. Once the run was completed, the gel was removed from the electrophoresis plates, and the loading wells were cut from the gel with a razor blade to make the gel dimensions 2” x 3.5”. A piece of polyvinylidene difluoride (PVDF, Thermo

Scientific, Rockwood, TN) membrane and 4 pieces of 1 mm filter paper were also cut to size. The PVDF membrane was primed in 100% methanol, following which the gel, membrane, and filter paper were placed into a glass dish containing approximately 200 mL 1X Tris-Glycine Transfer Buffer [48 mM Tris Base, 39 mM Glycine, 1.3 mM SDS, 20% (v/v) Methanol] and allowed to equilibrate for 20 minutes.

The protein was transferred to the PVDF membrane *in situ* using a "sandwich" set-up (Fig. 4) in a Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad, Hercules, CA). The transfer was conducted at 15 volts for 20 minutes, during which the protein bands were permanently fixed to the solid membrane for further manipulation.

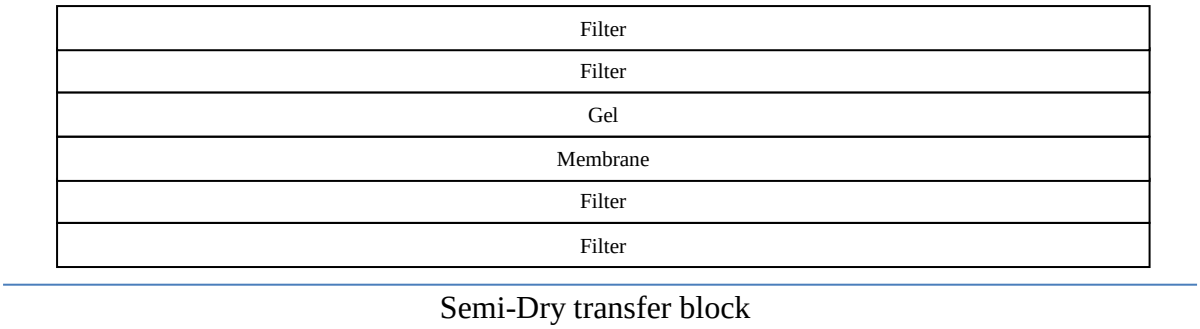

Fig. 4. "Sandwich" set-up for transferring protein from a polyacrylamide gel to a PVDF (polyvinylidene fluoride) membrane. Electric current runs through the sandwich from the top to the bottom, carrying the proteins from the gel and fixing them to the PVDF membrane directly underneath.

After electrophoresis, the membrane was removed from the sandwich using forceps and placed in a 50 mL solution of 5% Blotto in 1X Phosphate Buffered Saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) overnight in a Tupperware™ container, shaking, at 4°C to block nonspecific binding of antibodies to the membrane. Following overnight blocking, the 5% Blotto solution was removed and the

membrane was rinsed with RO H_2O and placed in a heat sealed plastic bag approximately 3" x 4" which was immediately sealed on 3 sides. Two mL of fresh 5% Blotto containing 20 µL anti-actin primary antibody (1:100 dilution) raised in rabbits (Sigma-Aldrich, St. Louis, MO) was placed into the bag, and the fourth side was sealed. The membrane was incubated in this primary antibody overnight, with gentle shaking, at 4°C. After approximately 16 hours, the membrane was removed from the bag using forceps, rinsed with RO H_2O, and placed in 50 mL of a 0.1% 1X PBS/Tween 20 solution to wash. This step was conducted at room temperature with shaking, and repeated twice with fresh 0.1% 1X PBS/Tween 20, rinsing the membrane thoroughly with RO H_2O between each wash to ensure complete removal of anti-actin antibody. Following the three wash steps, the membrane was placed into a 50 mL conical tube using forceps, so that the side containing the protein was facing inwards to assure contact with the solution to be added. Ten mL of fresh 5% Blotto containing 4 µL Goat anti-rabbit alkaline phosphatase conjugated secondary antibody (1:2500 dilution, Bio-Rad, Hercules, CA) was added to the conical tube. Proteins on the membrane were exposed evenly to the secondary antibody by rotating the conical tube at room temperature for 1 hour. Following this incubation, the membrane was removed from the tube using forceps and washed 3 times with 50 mL fresh 0.1% 1X PBS/Tween 20, rinsing the membrane thoroughly with RO H_2O between each wash. After the third wash, the membrane was laid flat ensuring no bubbles, protein-side facing upwards on a large piece of Saran™ plastic wrap. Two mL Immun-Star™ AP Substrate (Bio-Rad, Hercules, CA) was added to the membrane and allowed to incubate at room temperature for 10 minutes, at which point the substrate was poured off of the membrane, and the Saran™ wrap was folded over the membrane with no bubbles. The membrane was exposed to x-ray film for 5 minutes, then the film was

developed using a Konica Minolta SRX-101A developer (Konica Minolta Medical & Graphic, Inc., Shanghai, China).

Using 35 µg of total protein in each lane, the actin bands were inconsistent in intensity. The actin band at 2 hpf was darker, while the bands in 4 hpf – 12 hpf were lighter. To standardize loading of protein relative to actin, the total protein amounts run through SDS-PAGE analysis were adjusted until the actin bands were approximately the same intensity (Table 1), and these amounts were used throughout the subsequent Western blots.

Table 1. Total amounts of protein from embryos at varying developmental stages loaded into polyacrylamide gel. Samples were electrophoresed for further analysis via Western blot.

Developmental Stage of Protein	**Total Protein Loaded**
2 hpf (hours past fertilization)	25 µg (micrograms)
4 hpf	40 µg
6 hpf	40 µg
8 hpf	40 µg
12 hpf	40 µg

To test for anti-m2lg antibody reaction, another 4-15% gradient polyacrylamide Mini-PROTEAN® TGX™ pre-cast gel was run and transferred as previously described. The membrane was then placed into 50 mL of fresh 5% Blotto solution and allowed to block overnight at 4°C with shaking. After blocking, the membrane was rinsed thoroughly with RO H_2O and placed in 50 mL fresh 5% Blotto solution containing 50 µL purified anti-m2lg primary antibody (1:1000 dilution v/v) and incubated at 4°C overnight with shaking. Following the primary antibody step, the membrane was washed and exposed to the goat

anti-rabbit alkaline phosphatase conjugated secondary antibody and developed as described previously. After the membrane was exposed to x-ray film and a sufficient image was obtained, the membrane was stored in Saran™ plastic wrap at RT. A negative control was performed by running an identical SDS-PAGE gel and blotting as described previously. All steps were performed identically and simultaneously with the exception of the use of an equal amount of pre-immune serum taken from the New Zealand white rabbit before injection of the m2lg peptide in the place of the primary antibody.

Peptide Competition Experiment

To provide further support for antibody binding properties, a peptide competition experiment was performed using the peptide obtained from Biosynthesis, Inc (Lewisville, TX). Three polyacrylamide gels containing embryo protein samples at 4 hpf, 6 hpf, and 8 hpf were run, transferred to separate PVDF membranes, and blocked in 5% Blotto/1X PBS solutions as described above for the Western blot experiment in separate containers. Before performing the primary antibody incubation, purified anti-m2lg was exposed to the unconjugated m2lg peptide at different concentrations for 4 hours at room temperature (Table 2). Peptide exposure was conducted under constant agitation in the form of rotation to allow the antibody to bind the peptide prior to membrane exposure. After peptide exposure, solutions containing antibody and peptide were suspended in 50mL 5% Blotto/1X PBS in separate containers. One membrane was placed in each container and allowed to incubate overnight at 4ºC with shaking. Subsequent washing, secondary antibody incubation, substrate activation, x-ray film exposure, and development steps were conducted as described above, with the membranes contained separately throughout.

Table 2. Concentrations of unconjugated m2lg (meis-2 linked gene) peptide exposed to purified anti-m2lg AB (antibody). Antibody, peptide, and PBS (phosphate buffered saline) solutions were mixed in 1.5mL (milliliter) microcentrifuge tubes and allowed to rotate for 4 hours at room temperature.

Treatment	Amount of Antibody	Amount of Peptide	1X PBS	Total Volume
No peptide	50 µL	0 µL	950 µL	1000 µL (1 mL)
1:1 ratio 1ºAB:Peptide	50 µL	50 µL	900 µL	1000 µL (1 mL)
1:10 ratio 1ºAB/Peptide	50 µL	500 µL	450 µL	1000 µL (1 mL)

Immunohistochemistry

Immunohistochemistry (IHC) experiments were performed on 15 µm embryo cross-sections to localize m2lg expression within the developing embryo. Embryos were obtained and staged as described previously. Embryos at 48 hpf were dechorionated manually using No. 5 Dumont forceps (DUMOSTAR, Williston, VA) before fixing. Fixing was conducted by submerging the embryos in 750 µL 4% paraformaldehyde (PFA) [1.3M PFA, 0.002N NaOH, 10% v/v 10X PBS (1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 18 mM KH_2PO_4)] in a 1.5 mL microcentrifuge tube (approximately 30 embryos per tube) with gentle shaking for 10 minutes. PFA was removed and replaced with 750 µL fresh 4% PFA, and embryos were incubated with gentle shaking for 16 hours at 4°C. After 16 hours, the PFA was removed and the embryos were washed by adding 750 µL 1X PBS and incubating with shaking for 10 minutes. Embryos at 48 hpf exhibit pigmentation which prevents

visualization of protein using antibodies, so they were depigmented using an H_2O_2 solution [3% H_2O_2, 1% KOH (Kim et al., 2012)]. Embryos were placed into a petri dish containing 7 mL H_2O_2 solution and allowed to depigment for 30-45 minutes, until pigmentation was fully removed. Following this, the embryos were removed using a glass pipette, and placed into a petri dish containing 1X PBS in order to remove bubbles. Embryos were then dehydrated for storage by washing twice for 10 minutes with 750 µL 50% methanol/1X PBS, followed by two 10 minutes washes in 100% methanol. Embryos were stored in 100% methanol at -20°C.

To rehydrate embryos for use in experiments, embryos were removed from microcentrifuge tubes using glass pipettes and placed at a maximum of 4 embryos per well into 12 well plates. Each well contained 150 µL of 100% methanol prior to the addition of the embryos to prevent them from drying. The embryos were then rehydrated by performing sequential 10 minute washes of 800 µL each of 75% methanol/1X PBS, 50% methanol/1X PBS, and 25% methanol/1X PBS, followed by two 10 minute washes with 800 µL 1X PBS, removing the previous solution before beginning each wash.

Following rehydration, embryos were prepared for sectioning. A 1.5% agarose gel containing 5% sucrose was dissolved in 1X PBS and poured into mini petri dishes. Before the gel solidified, 2-3 embryos were placed into the petri dish and positioned perpendicularly, with their anterior regions facing downwards, using needle probes. Once gels containing the positioned embryos were solidified, they were placed at 4°C for a minimum of 15 minutes to ensure the gel was solid. Embryos were then cut out of the gel in 5 mm x 5 mm blocks and placed in a large petri dish containing a solution of 30% sucrose in RO H_2O and kept at 4°C for a minimum of 16 hours and a maximum of 5 days. Embryo blocks were then positioned

with the anterior region facing downwards in the center of aluminum foil wells (Fig. 5), submerged in Optimal Cutting Temperature (O.C.T.,Tissue-Tek®, Torrance, CA) compound, and frozen by placing the well on a block of dry ice. Embryo blocks were stored at -80°C. Embryo blocks were cut in 15 µm cross sections through the eyes and brain using a Leica CM-1100 Bench Cryostat (Leica Microsystems, Buffalo Grove, IL). Sections were placed onto VistaVision™ HistoBond ® Adhesive Slides (VWR, Radnor, PA) to permanently bind the tissue onto the slide. Slides were stored at -20°C.

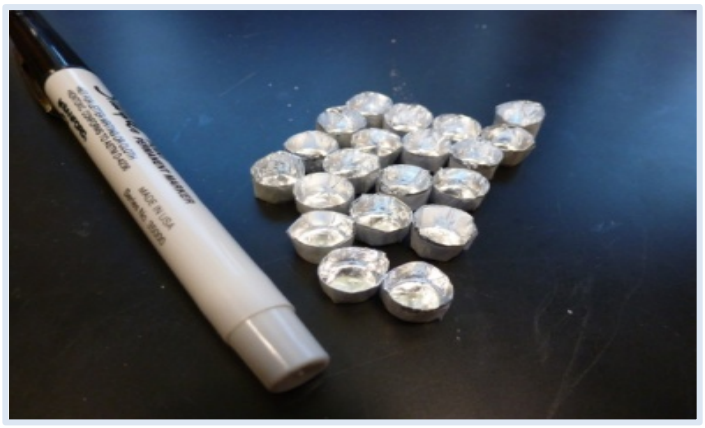

Fig. 5. Aluminum foil wells for preparing embryo blocks for sectioning. Wells were constructed by wrapping 2.5 cm x 2.5 cm pieces of aluminum foil around the flat end of a fine tipped Sharpie, using a 0.5 cm strand of tape to hold the well's shape, and cutting off the excess with scissors. Embryo blocks were placed in the center of the wells, which were then filled with O.C.T. (Optimal Cutting Temperature) compound, and placed on top of a block of dry ice to ensure freezing.

Sections were rehydrated for immunohistochemistry by adding 1 mL 0.3% 1X PBS/Triton X-100 and allowing each slide to soak for 5 minutes, then draining slide on a paper towel. Slides were blocked for 30 minutes at room temperature with 200 µL blocking

solution [2% dimethyl sulfoxide (DMSO), 5 mg/mL BSA, 5% Normalized Goat Serum]. After 30 minutes, the blocking solution was drained onto a paper towel via capillary action, and 500 µL primary antibody solution was added to the slide (1:100 purified anti-m2lg antibody diluted in blocking solution) and allowed to soak overnight at room temperature in a closed chamber to prevent evaporation of the antibody solution. Negative controls were exposed to a 1:100 solution of pre-immune serum diluted in blocking solution under the same conditions. The antibody solution was then drained onto a paper towel and the slide was washed 3 times by soaking with 250 µL 0.3% 1X PBS/Triton X-100 for 5 minutes each, draining wash solution on a paper towel after each wash. The slide was then incubated with 500 µL of a 1:5000 dilution Alexa-Fluor® 488 goat anti-rabbit IgG (Invitrogen, Grand Island, NY) in 0.3% 1X PBS/Triton X-100 for 1 hour at room temperature, followed by three more 5 minute washes with 250 µL 0.3% 1X PBS/Triton X-100, draining solutions onto a paper towel after each step. Following the wash steps, a coverslip containing a thin layer of ProLong® Gold Antiface Reagent (Invitrogen, Grand Island, NY) was placed over the slide to preserve fluorescence, and slides were stored at 4°C in a dark container until imaging. Slides were inverted and imaged using a Zeiss Confocal Laser Scanning Microscope 510 (Leica Microsystems, Buffalo Grove, IL).

RESULTS

Multiple Sequence Alignment

The multiple sequence alignment aligned the predicted protein sequences of M2lg from zebrafish (*Danio rerio*), mouse (*Mus musculus*), human (*Homo sapiens*), and chicken (*Gallus gallus*). The 300 aa sequence from zebrafish aligned with a 281 aa sequence from mouse (61% conserved identity to zebrafish), a 281 aa sequence from human (70% conserved identity), and a 272 aa sequence from chicken (59% conserved identity). The predicted m2lg protein sequence from zebrafish possessed an extra 18 residues on the N-terminal region that are not present in the mouse, human, or chicken sequences, which may indicate a gain of function domain in zebrafish that is not present in the other organisms. Conversely, the chicken M2lg sequence seems to have undergone a deletion and is missing 11 residues that are present in the other three sequences. Multiple regions within the sequence show a high level of identity, with 9 regions possessing 100% identity over 5 or more amino acids. These regions may be a product of evolutionary pressure and likely indicate functional domains of the protein; however, the sequences do not match any known protein domains that have been characterized to date (Fig. 6).

Fig. 6. Multiple sequence alignment of M2lg (Meis-2 linked gene) predicted protein sequence in zebrafish [*Danio rerio* (Drm2lg), 300 aa (amino acids)], mouse [*Mus musculus* (Mmm2lg), 281 aa], human (*Homo sapiens* (Hsm2lg), 281 aa], and chicken (*Gallus gallus* (Ggm2lg), 272 aa]. Red blocks indicate amino acids that are identical among all four species. The region encompassed within the black square was determined by Biosynthesis, Inc. (Lewisville, TX), to be the region of the sequence that was most likely to elicit a sufficient immune response in rabbit. The blue underlined region indicates the sequence from zebrafish that was synthesized by Biosynthesis, Inc., and further used to inject into a New Zealand white rabbit.

Antibody Generation

The multiple sequence alignment was sent to the company Biosynthesis, Inc. (Lewisville, TX), where it was determined that amino acids 232-250 represented a peptide

sequence most likely to illicit an immune response when injected into a rabbit. In addition, this sequence is highly conserved among vertebrates, which we felt would increase our chances of producing a cross-species reactive antibody (Fig. 6). This 19 aa sequence was synthesized and delivered to our lab in an unconjugated form (peptide only) at a concentration of 0.2 mg/mL, as well as conjugated to Keyhole Limpet Hemocyanin to ensure a high yield of antibody production at a concentration of 1.01 mg/mL.

Antibody Purification

Following purification of the anti-m2lg antibody from total rabbit serum, purified eluates were run on an SDS-PAGE gel, along with the unpurified serum as well as samples from each wash step, to confirm purification. The presence of two distinct bands at approximately 50 kDa (kiloDalton; heavy chain) and 25 kDa (light chain) indicated successful antibody purification, whereas large darkly stained areas on the gel indicated unpurified serum (Fig. 7).

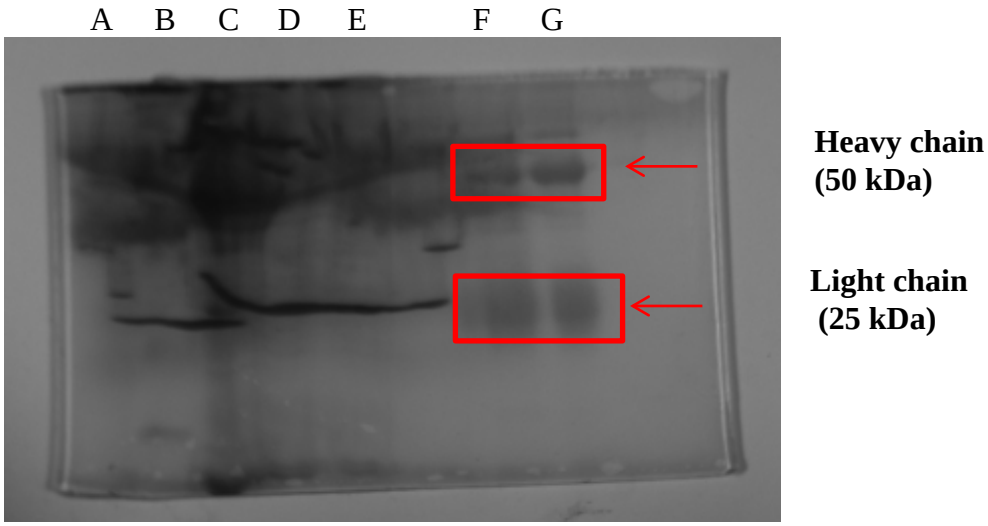

Fig. 7. Coomassie stained SDS-PAGE gel analyzing purification of antibody. Lanes A-E contain unpurified filtered rabbit serum, diluted unpurified serum, and three wash solutions containing residual proteins, respectively. The large amounts of protein present in the gel in these lanes caused no visible banding patterns. Lanes F and G contain eluted antibody. The two distinct bands present in these lanes represent the heavy chain and the light chain of the antibody and

Western Blot

Western blot using the purified anti-m2lg antibody to probe for the zebrafish m2lg protein resulted in the presence of a specific band across various stages of development (Fig. 8.). Three identical gels were run as previously described. The experimental treatment comprised probing one blot with the purified anti-m2lg antibody. In contrast, the negative control comprised using total pre-immune serum from the rabbit used in the antibody generation. The pre-immune serum, extracted at week 0 of the antibody generation experiment, was not exposed to the peptide antigen and, therefore, did not contain antibodies against it. The loading control treatment was probed with an antibody against zebrafish actin protein to verify that all protein loading was consistent.

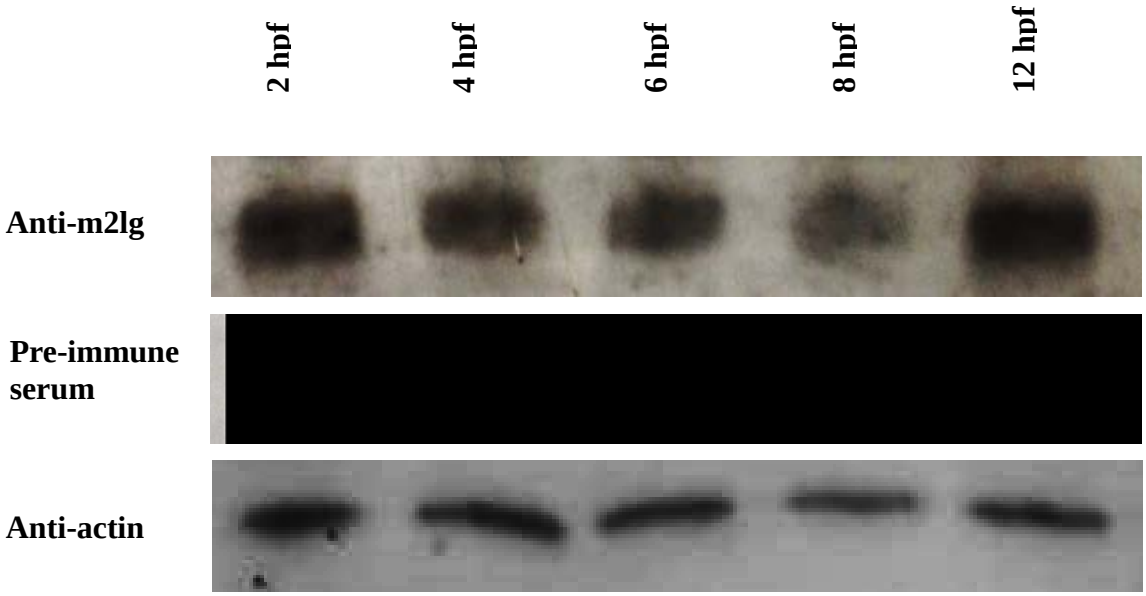

Fig. 8. Western blot of total embryo protein samples from zebrafish embryos at various developmental stages. In the experimental treatment [Anti-m2lg (meis2-linked gene)], a band is present at 58 kDa (kiloDalton) from 2 hpf (hours past fertilization) to 12 hpf. No bands are present at 58 kDa in the negative control (Pre-immune serum) treatment. In the loading control lane (Anti-actin), a band is present at 46 kDa that remains consistent across all time stages, demonstrating consistent protein loading.

After the blots were performed, a band approximately 58 kDa in size was present in the experimental treatment. Interestingly, the predicted size of the zebrafish m2lg protein is 34 kDa. This band was very distinct at all developmental stages tested. The band was not present in the negative control blot probed with pre-immune serum, however, indicating that the anti-m2lg antibody generated and purified from rabbit was specific to the 58 kDa protein as shown in Fig. 8. The loading control blot probed with the anti-actin antibody showed a band at approximately 46 kDa that was present and consistent in intensity throughout the developmental stages. Because actin is expressed throughout development at relatively

constant levels, this indicates that the amount of protein loaded for each stage was consistent. Taken together, these data indicate that a 58 kDa protein is specifically recognized by our anti-m2lg antibody and is expressed and active early in development.

Peptide Competition Experiment

Due to the discrepancy between the predicted m2lg protein size and the size of the band picked up by the anti-m2lg antibody, a peptide competition experiment was performed to determine antibody binding specificity (Fig. 9).

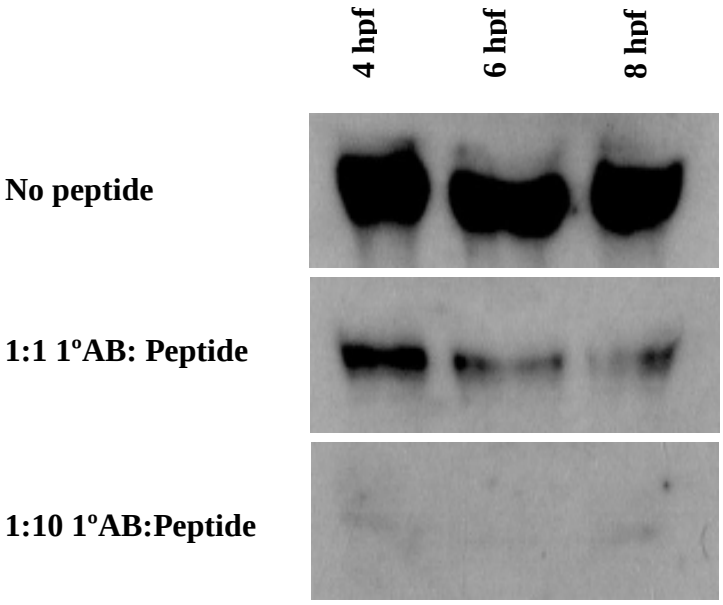

Fig. 9. Peptide competition experiment to determine antibody specificity against 58 kDa (kiloDalton) protein. Western blot treatment of protein with anti-m2lg (meis2-linked gene) antibody alone (top) at 4, 6, and 8 hpf (hours past fertilization). Treatment containing equal volumes of antibody and m2lg peptide (middle). Treatment containing 10 times more volume of peptide than antibody (bottom).

The blot exposed to purified anti-m2lg primary antibody alone (no m2lg peptide added) showed dark bands at 58 kDa in every stage tested. The blot exposed to the treatment containing a 1:1 v/v ratio of anti-m2lg antibody and m2lg peptide also contained bands at 58 kDa in every developmental stage that was tested. Although still present, the bands appeared fainter, indicating that a portion of the anti-m2lg antibody was bound by the peptide and was therefore unavailable to subsequently bind to the protein on the membrane. The final treatment, during which the anti-m2lg antibody was exposed to 10 times more total volume of m2lg peptide, produced no visible bands at 58 kDa. This indicated that the anti-m2lg is specific to the peptide portion of m2lg against which it was raised.

Immunohistochemistry

IHC using total rabbit serum was performed to determine localization of protein expression within the developing embryo. IHC on transverse cross-sections through the eye and head region of a zebrafish embryo at 48 hpf showed distinct antibody binding in various areas (Fig. 10). The strongest expression was observed in the developing retina and optic nerve (Fig. 10 A-B). This expression is absent in the negative control IHC using pre-immune serum (Fig. 10 C), indicating that the peptide is highly expressed in this region. Interestingly, expression of m2lg is present in the optic nerve and the inner nuclear layer of the retina, but is conspicuously absent from the lens and the photoreceptor cell layer of the eye (Fig. 10 B). The apparent expression within the outer dermal layer of the embryo is also observed in the negative control (Fig. 10 C). Therefore, this expression is unlikely to represent actual m2lg expression and may be due to other proteins contained within the serum or the natural autofluorescence of zebrafish.

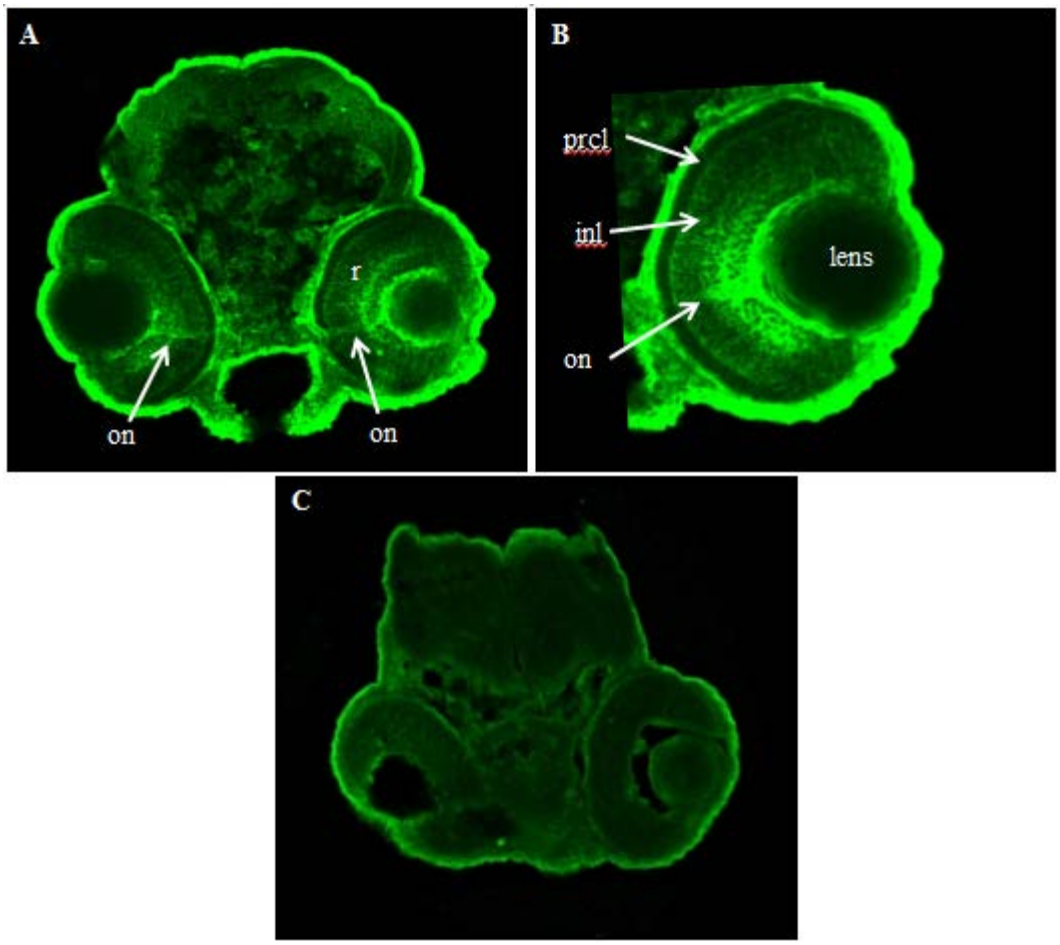

Fig. 10. IHC (Immunohistochemistry) of 48 hpf (hours past fertilization) embryo cross-sections using total rabbit serum. (A) IHC using total rabbit serum containing anti-m2lg (meis2-linked gene) antibody expression within the retina (r) and optic nerve (on) of the zebrafish. (B) Cross-section of the eye expressing m2lg in the optic nerve (on), the inner nuclear layer (inl), and the photoreceptor cell layer (prcl). (C) Negative control IHC using pre-immune serum from the rabbit instead of anti-m2lg antibody.

DISCUSSION

To date, little is known about the novel zebrafish gene *zgc:154061*, temporarily named *m2lg* (*meis2 linked gene*). Orthologs and putative orthologs have been found in all vertebrates with publicly available genome data as well as in urochordates, cephalochordates, and echinoderms. In vertebrates, *M2lg* is always present downstream of *Meis2* (*meis2.2* in zebrafish) in an inverted, convergently transcribed orientation. In the nematode worm *C. intestinalis* the two genes are not adjacent to one another, and in amphioxus they are adjacent but appear to be transcribed in the same direction (Carpenter, 2010). The high degree of conservation of this gene in sequence and genomic organization relative to *Meis2* indicates a high level of evolutionary pressure on its genetic linkage to *Meis2* and demonstrates its ancient deuterostome origins. In this study, we have taken steps towards characterizing various aspects of this gene's expression profile in addition to the work done by Carpenter (2010) and Graham (2009).

The multiple sequence alignment illustrates the high M2lg sequence identity shared among humans, mice, chickens, and zebrafish. Evolutionarily speaking, the high degree of sequence conservation suggests that the gene in question plays some important role for the organism. Additionally, within the M2lg protein sequence alignment, there are multiple regions of 100% identity shared among all four organisms compared, indicating a higher level of selective pressure to maintain these regions. This suggests that these regions may be conserved protein domains that are performing critical functions for the gene. To date, however, no domains have been characterized in other known proteins that match these

conserved regions. If these regions are functional domains, the role(s) that they play within the organism have yet to be described.

To characterize the spatial and temporal expression of a protein within an organism, an antibody against that protein is necessary in order to localize and identify it among the numerous other proteins present. In the case of an uncharacterized protein such as this one, no antibody existed previously with which to carry out the required experiments. To this end, we generated an antibody against a small peptide portion of the m2lg protein in zebrafish using a New Zealand white rabbit. Once the rabbit was exsanguinated, the serum was purified for IgG antibodies, which are produced in response to repeated antigen exposure, in contrast to the more general IgM proteins produced as a rapid response to a single exposure (Geisberger et al., 2006; Goding, 1978). As the only antigen the rabbits should have been exposed to was the m2lg peptide, the only IgG antibodies present within the serum should have been against this peptide. After purification, SDS-PAGE analysis showed the presence of two distinct bands within the purified antibody lanes which correlate to the general sizes of the heavy chain and light chain of an antibody. This indicated that IgG antibodies were present in the immune serum of the rabbit and that the purification steps resulted in a sample containing only the antibodies that were generated.

Following purification, the antibody was used in Western blots to determine whether the antibody would recognize and bind specifically to the m2lg protein. The Western blot performed on developmental stages spanning 2-12 hpf using the purified antibody resulted in the presence of a band at 58 kDa. The presence of a single band rather than numerous bands or a solid smear of protein indicates that the antibody generated in house is specific to a single protein that is expressed within the developing zebrafish embryo. Furthermore, this

band was absent in the blot performed using pre-immune serum. The pre-immune serum was total serum taken from the rabbit immediately prior to the first m2lg peptide injection. As a result, it contained identical proteins to the serum obtained post-exposure with the key absence of the antibody generated during the m2lg exposure. Therefore, the absence of the 58 kDa band in the pre-immune serum treatment indicated that a protein of this size was successfully recognized by an antibody that was generated against the peptide portion of the m2lg protein in zebrafish.

The size of the predicted protein translated from the open reading frame *m2lg* was approximately 34 kDa, which is 24 kDa smaller than the 58 kDa size of the protein recognized by the anti-m2lg antibody. This difference between predicted and observed size of the protein poses a dilemma. In order to determine whether the antibody was errantly binding a different protein than the zebrafish m2lg, a peptide competition experiment was performed. By incubating the antibody with the m2lg peptide prior to membrane exposure, we tested whether the antibody was specifically binding to the peptide or to another protein. As the amount of peptide incubated with the antibody was increased, the band at 58 kDa became increasingly more faint. When the anti-m2lg antibody was incubated with 10 times the volume of peptide, the band almost completely disappeared. This absence of the 58 kDa band indicated that the antibody was completely bound to the peptide during the competition experiment and was therefore no longer available to bind to the protein on the membrane, providing further evidence for its specificity.

The presence of a 58 kDa protein rather than the expected 34 kDa protein likely indicates the occurrence of post-transcriptional modification of the m2lg protein in zebrafish. One possibility that may explain the size discrepancy observed is glycosylation of m2lg, a

process which covalently adds a glycan structure to the backbone of the protein structure in order to alter its function (Imperiali and O'Connor, 1999). This agrees with the presence of a larger band as covalent bonds persist while typical protein-protein interactions are denatured during the Western blot process. Glycosylation typically aids in increasing the stability of the protein and the absence of the proper glycosylation proteins has been shown to cause numerous defects in development, oftentimes affecting muscle or nervous system development (Freeze et al., 2012; Jaeken and Carchon, 1993). As *m2lg* has been shown previously to be expressed in the developing brain, this provides a possibility for the observed increase in protein size. More work is currently being done to elicit the exact cause by immunoprecipitating the entire protein and performing Mass Spectrometry analysis. This information will likely be useful in determining the function of this novel gene.

IHC experiments performed in this study show that m2lg is present in the developing zebrafish at 48 hpf. Its expression, while clearly visible, is largely restricted to the developing retina at this stage and is distinctly present within the developing optic nerve. These findings suggest a potential role for *m2lg* during development of the eye. Furthermore, this expression partially overlaps with *meis2.2* expression in zebrafish, which is also present in the developing eye fields (Waskiewicz et al., 2001). These similarities in expression patterns indicate that *m2lg* may be under similar regulatory control to *meis2.2*, with the potential that the two genes may be sharing a regulatory element that has been located downstream of *meis2.2* within an intron of *m2lg* (Nelson, 2011).

Taken together, these data provided in this study concerning the expression patterns of m2lg in zebrafish along with work done by previous members of the Zerucha lab indicate that *m2lg* is possibly part of a GRB that also includes *meis2*. It is possible that *m2lg* is a

bystander gene of the GRB, but it may also share regulatory control with *meis2*. Research is currently being conducted within our laboratory to elicit the function of this gene and determine whether the putative regulatory element is controlling one or both of the genes present within the proposed GRB. The work done within this study lends information towards characterizing a novel, previously undescribed gene that, while not yet fully understood, is likely playing some conserved role in vertebrate development.

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VITA

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